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CHARACTERIZATION OF THE P. BREVIS POLYETHER NEUROTOXIN BINDING COMPONENT IN EXCITABLE MEMBRANES

FINAL REPORT

Daniel G. Baden and Thomas J. Mende

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The brevetoxins bind to a unique receptor site associated with excitable membranes. The site, which possesses a Kd of 2.6-3.3 nM and a Bmax of 5.75-6.0 pmoles/mg protein, is present in rat, fish, and turtle synaptosomes. This high affinity, low capacity site, is accompanied by a lower affinity, higher capacity site which is currently under investigation. The high affinity site is known as site 5, and binds toxin with half-maximal affinity and avidity at concentrations which yield half-maximal 22Na ion influx and half-maximal nerve membrane depolarization. Toxin binding at this high affinity site is membrane potential independent and does not interact with any of the previously described sodium channel binding sites. The binding characteristics of the lower affinity site correlate well with observed allosteric modulation observed in sites 1-4 of the voltage-sensitive sodium channel. Photoaffinity probes incorporating brevetoxin in a covalent form displace brevetoxin from the binding site of high affinity, underscoring their potential as specific

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...probes for purification of the site. Both size chromatography and brevetoxin-affinity columns, following solubilization of the site from membranes holds promise for isolation, purification, and characterization of the specific site. The Scope of Work outlined in the original contract has been completed.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) have adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication (NIH) 86-23, revised 1985)).

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I. List of Appendices, Illustrations, Tables

II. Statement of the Problem

The research summarized herein was aimed at characterization of the binding site for *Ptychodiscus brevis* neurotoxins in nerve tissues. To achieve this objective, we have performed the following tasks:

[1] prepared synaptosomes from rats, fish, and turtles according to established procedures;

[2] determined the degree of toxin PbTx-3 binding to each species synaptosomes using tritiated PbTx-3 as specific probe, and have determined apparent dissociation constants (K_d) and binding maxima (B_{max});

[3] determine, using the rat system, on- and off-rates of binding, reversibility of binding, and binding under depolarizing conditions;

[4] determined the degree of displacement of labeled PbTx-3 by other naturally-occurring and synthetic derivative of brevetoxins in each species synaptosomes;

[5] determined the degree of displacement of labeled brevetoxn from its specific site (Site 5) by natural toxins specific for specific binding sites 1-4;

[6] characterize each potential competitor in terms of competitive, non-competitive, uncompetitive, mixed, or lack of inhibition pattern;

[7] construct and examine brevetoxin photoaffinity labels for displacing ability in the rat system.

For purposes of this narrative summary of the work performed, each task enumerated above will be treated under separate headings.

III. Summary of Results Generated

A. Synaptosome Preparation

Excitable tissue preparations were obtained fresh daily from live animals using the techniques described by Dodd *et al.* (1). Frozen synaptosomes (-80°C) were useful when a large series of experiments was to be performed; synaptosomes from several animals could be pooled to provide a reasonable tissue correlation for several days experiments. Non-specific binding was higher in these preparations, however. Protein was measured on each synaptosome preparation using the Bradford technique (2).

Synaptosomes from rats, turtles, or fish are prepared in approximately the same manner with minor exceptions. Turtle synaptosomes are slightly more dense than are rat synaptosomes, and are collected at a 0.32M/1.2 M sucrose interface following ultracentrifugation. Fish synaptosomes require the addition of 370 mM sucrose during ultracentrifugation and during experiments to maintain iso-isomolarity with fish serum. In contrast to turtle and rat synaptosomes, fish synaptosomes do not demonstrate specific binding beyond the P_2 step during purification.

B. Measurement of Labeled Toxin Binding

1. Tritiated Toxin Synthesis. Tritiated sodium borohydride, NaB^3H_4, is available at specific activities approaching 80 Ci/mmole. Reduction of the α,β-unsaturated aldehyde function in PbTx-1 or PbTx-2 results in PbTx-7 and PbTx-3, respectively, with specific activities

25% that of the reducing reagent (ca. 20 Ci/mmole). A reduction of the α-methylene in either PbTx-7 or PbTx-3 results in toxins with specific activities twice that of the primary reduction toxins (ca. 40 Ci/mmole).

2. Binding Assays. Binding of tritiated PbTx-3 was measured using a rapid centrifugation technique. All binding experiments were conducted in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/mL bovine serum albumin, and 0.01% Emulphor EL-620 as an emulsifier (3). In addition, 370 mM sucrose was added to fish synaptosome experiments to maintain iso-osmolarity (4).

Synaptosomes, suspended in 0.1 mL of binding medium minus BSA, were added to a reaction mixture containing [^{3}H] PbTx-3 and other effectors in 0.9 mL of binding medium in 1.5 mL polypropylene microfuge tubes. After mixing and incubating at the desired temperatures for 1 hr, samples were centrifuged at 15,000 x g for 2 min. Supernatant solutions were sampled for the measurement of free toxin concentrations, and the remainder was aspirated in each case. Pelleted synaptosomes were rapidly washed with 4 drops of a wash medium consisting of 5 mM HEPES (pH 7.4), 163 mM choline chloride, 1.8 mM calcium chloride, 0.8 mM magnesium sulfate, and 1 mg/mL BSA. Pellets were transferred to liquid scintillation vials containing 3 mL of liquid scintillant, and the bound radioactivity was measured using liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 and was subtracted from total binding to yield specific binding. Dissociation constants and binding maxima are summarized in Table I.

Table I. Comparison of Dissociation Constant (K_d) and Binding Maximum (B_{max}) in Fish, Turtles, and Rats*

Species	K_d (nM)	B_{max} (pMol/mg protein)	Temp. Optimum (°C)	Specific Binding at K_d
Fish	6.1	1.40	23	80%
Turtle	1.5	2.25	4	80%
Rat	2.6	6.80	4	90%

*mean values for K_d and B_{max}, n=9,4,6 for fish, turtles, and rats respectively.

A preliminary comparison of specific binding of tritiated PbTx-3, PbTx-7, PbTx-9, and PbTx-10 indicates an equivalent B_{max} and a progression of K_d values which parallel the relative potencies of the labeled brevetoxins. This is a further indication to us that binding affinity is the conservative requirement in the potency of the brevetoxins (Table II), and further, that we may be able to utilize the toxins which are of higher specific activity for more detailed receptor characterization (5).

Table II. Comparison of K_d and B_{max} for Four Different Tritiated Brevetoxin Probes

Toxin	K_d (nM)	B_{max} (pmoles/mg protein)
PbTx-3	2.13	6.99
PbTx-9	8.76	6.75
PbTx-7	1.91	6.38
PbTx-10	1.56	6.46

C. On- and Off- Rates, Reversibility of Binding, and Binding under Depolarizing Conditions

Our evidence indicates that, at a K_d concentration of tritiated PbTx-3, the $t_{1/2}$ for on- and off-rates approximate 1-2 minutes. A closer approximation cannot be derived utilizing present protocols. There is no membrane potential dependence of brevetoxin binding to the high affinity, low capacity binding site known as Site 5. K_d = 2.6 (intact), 2.9 (lysed), 3.3 (depolarized) and B_{max} = 6.01 (intact), 5.83 (lysed) and 5.75 pmoles/mg protein (depolarized) (6).

D. Degree of Displacement of Labeled PbTx-3 by Other Naturally-Occurring and Synthetic Brevetoxin Derivatives

Regardless of the organism used for synaptosomal preparations, it is apparent to us that the topographic characteristics of the brevetoxin binding site on the VSSC are comparable. Using brevetoxins PbTx-1, -2, and -3, IC_{50} data for specific displacement of tritiated PbTx-3 shows comparable data in each case (Table III). The more hydrophobic type-2 brevetoxins are most efficacious in their ability to compete for site 5 binding (7). It is of interest to note that ciguatoxin is thought to resemble brevetoxin-A.

Table III. Specific Displacement of [^{3}H] PbTx-3 from Synaptosome Binding by Unlabeled Brevetoxins, Comparison with LD_{50}

Toxin	Competitor Toxin Concentration (nM)			
	Turtle (1) (IC_{50})	Fish (2) (IC_{50})	Rat (3) (IC_{50})	Fish (LD_{50})
PbTx-1	3.0	30	3.5	4.4
PbTx-2	10.3	70	17.0	21.8
PbTx-3	15.0	110	12.0	10.9
PbTx-5	----	---	13.0	42.5
PbTx-6	----	---	32.0	35.0
PbTx-7	----	---	4.1	4.9

Tritiated toxin concentrations were (1),(3) 10.0, and (2) 12.0 nM.

Inhibition constants have been determined in each of the cases indicated in Table IV above by utilizing the K_d concentration of tritiated PbTx-3, and the following equation:

$$K_i = IC_{50}/(1+C/K_d),$$

where K_i = the inhibition constant, IC_{50} = the 50% inhibition concentration of the competitor, C= tritiated PbTx-3 concentration, and K_d is the dissociation constant of the lab (Table IV).

Table IV. Inhibition Constants for Derivative Brevetoxins Derived from the Cheng-Prusoff* Equation

Toxin	K_i (nM) Turtle	Fish	Rat
PbTx-1	0.39	10.10	0.72
PbTx-2	1.34	23.57	3.51
PbTx-3	1.96	37.04	2.47
PbTx-5	----	----	2.68
PbTx-6	----	----	6.60
PbTx-7	----	----	0.85

*see discussion under heading F. of this report. K_i derived in this manner require pure competitive inhibition for validity.

E. Inhibition of Brevetoxin Binding by Natural Site 1-4 Toxins

There was no difference in brevetoxin binding in the presence of Site 1 (tetrodotoxin or saxitoxin), Site 2 (veratridine or batrachotoxin), Site 3 or 4 (the scorpion toxins) (3). Thus, the Site 1-4 natural toxins are classified as <u>not</u> competing with Site 5, the brevetoxin binding site. However, the two structural backbones of brevetoxin appear to differentiate between a high affinity, low capacity---and a lower affinity, high capacity binding site. It is this latter site, we believe, that has been often implicated in allosteric modulation in sodium channel binding by natural toxins.

F. Delineation of Two Brevetoxin Binding Sites

During the contract period, we have become increasing unsettled with respect to two apparently contradictory sets of information. First, the brevetoxins bind with an affinity constant which is consistently in the 1-5 nM concentration range, in good agreement with affinity data for other potent marine toxins like saxitoxin (6). In addition, the binding maximum in synaptosomes is also in good agreement with data for Site I toxins, which are known to bind to channels with a 1:1 stoicliometry. However, the allosteric modulation of sodium channel binding by other natural toxins by brevetoxins occurs at brevetoxin concentrations much higher, ca. 20-100 nM (9). This data is inconsistent with high affinity, low capacity binding (10).

Converse to this allosteric modulation which occurs at higher brevetoxin concentrations, is the finding that membrane depolarization (11), ^{22}Na (3) influx and <u>competitive</u> displacement of tritiated brevetoxin binding by unlabeled competitors, is dose dependent in the

same concentration ranges observed for the high affinity binding site (7). Thus, the allosteric modulation at other sodium channel binding sites appears to arise from brevetoxin interaction with a lower affinity, high capacity binding site.

Using classical Rosenthal analysis, we have been able to distinguish two separate specific brevetoxin sites (Table V).

Table V. The Two Brevetoxin Binding Sites

Site	K_d	B_{max}	Allosteric Modulator
5	2.6-3.3	5.7-6.8	No
_*	79.1-300.	6[illegible]/-180	Yes

*not numbered until further work can be accomplished.

The two site hypothesis is supported by brevetoxin inhibition constant data and double reciprocal competition plots, which indicate a deviation from competitive type patterns to non-competitive type patterns at higher competitor brevetoxin concentrations. The non-competitive displacement appears to be specific in nature, and is not likely due to changes in membrane fluidity. But, certainly more investigation is required before concrete conclusions can be offered.

G. Photoaffinity Probe Synthesis and Specific Displacement Experiments

We have succeeded in producing two photoaffinity probes which can be covalently attached to toxin PbTx-3. These two compounds are: PbTx-3-*p*-azidobenzoyl ester and PbTx-3-*p*-hydroxyphenyl-3-*p*-azidophenyl propionyl ethylene diamine monoamide. The former compound should be amenable to tritiation of the PbTx-3 moiety prior to coupling; the latter compound is amenable to pre-tritium labeling of the toxin moiety or post-radioactive iodine labeling in the ring of the photoaffinity portion of the coupled probe. Both compounds possess inhibition constants for displacment of tritium labeled PbTx-3 from its specific binding site in rat brain synaptosomes of 3.09 and 10.3 nM, respectively. Thus, we conclude they bind to Site 5 with sufficient affinity to be of use to us in identifying the brevetoxin binding component.

In the latter case, we believe we have produced a toxin derivative with solubility characteristics close to unaltered PbTx-3, and hence will be of greater use in identifying the binding component in intact synaptosomes. A minor problem was encountered in producing high yield products from the toxin coupling to affinity probe, however, in subsequent experiments. This was solved by protecting the phenolic group on the photoaffinity portion of the molecule to prevent self-condensation (the coupling function on the toxin PbTx-3 being an alcohol, also). Protecting with a cyclohexyl functionality, with subsequent cleavage after brevetoxin condensation, appears to provide the solution. These reactions are currently being evaluated, and products are being spectroscopically anaylyzed by FT-IR to provide the evidence for structure integrity and indentity.

II. Solubilization of the Brevetoxin Binding Site

1. Solubilization. For purposes of Site 5 purification from rat brain synaptosomes, it was necessary to solubilize the membrane bound protein with detergent. Frozen synaptosomes in 5 mL synaptosome binding medium (15)(-80°C) from a single rat brain were thawed and centrifuged at 130,000 x *g* for 35 minutes. The pellet was resuspended in 2.5 mL of a solubilization medium consisting of: 100 mM choline chloride, 20 mM HEPES (pH 7.4), 0.05% egg phosphatidyl choline, 0.1 mM PMSF, 1 mM iodoacetamide, 0.001 mM pepstatin A, and 1 mM o-phenanthroline. Over a period of 20 minutes, 0.25 mL aliquots of 4% Triton X-100 were added until 2.5 mL total detergent had been added. The resulting suspension containing solubilized membrane components was centrifuged at 150,000 x *g* for 40 minutes, and the supernatant solution was saved. A portion was examined for specific binding activity using tritiated PbTx-3. To the remainder of the supernatant solution was added 10mM final concentration of calcium chloride.

2. Column Chromatography. Sephacryl S-300 was packed in a 1 cm x 37 cm column and was washed with two bed volumes of mobile phase consisting of: 0.1% Triton X-100, 0.02% egg phosphatidyl choline, 50 mM choline chloride, 10 mM HEPES, 10 mM calcium chloride, 0.02% sodium azide, and the protease inhibitors at concentrations used for solubilization. The flow rate was adjusted to 2 psi, and the column was standardized. Solubilized synaptosomes were loaded on the column and fractions were collected from void volume to total volume. Bradford protein and specific brevetoxin binding activity was assessed in each fraction using the binding protocol established for brevetoxin radioimmunoassays (16), another solubilized specific brevetoxin binding component. The fractions which bind tritiated PbTx-3 in a specific manner will be pooled and subjected to purification by brevetoxin affinity column chromatography using the column matrix described in the previous section.

3. Status of Purification. The results of coupling reactions indicate that we were successful in coupling PbTx-3 to AH Sepharose 4B. Using a small amount of tritiated PbTx-3 in the reaction mix as tracer, we calculate about a 25% efficiency in coupling. We are currently attempting to improve on efficiency.

Preliminary results employing the afifnity column deal solely with brevetoxin specific antibodies as specific binding model system. The results indicate that we can pass crude IgG solutions through the column, wash with excess phosphate buffer (until no protein is detected in eluent) and then simply strip brevetoxin-specific antibodies from the column with 3 M NaCl solution. We feel the coupling is a success and that the affinity column feasibility has been demonstrated. We have successed in demonstrating that solubilized membrane protein from rat brain synaptosomes can be separated on Sephacryl A-300 columns (size fractionation). Of the fractions collected, significant specific binding of tritiated brevetoxin PbTx-3 was seen in fractions 30-37. Similarly, significant amounts of protein were demonstrated in the same fractions. The size to which this large protein peak corresponds is in the 230,000 to 350,000 dalton molecular weight range. This suggests that the brevetoxin binding component interacts with the α-subunit of voltage-sensitive sodium channels in rat brain synaptosomes. There is little specific brevetoxin binding in any other smaller molecular weight range, further illustrating that brevetoxins

do not likely interact with either of the β-subunits. This finding, coupled with our already known facts about specific brevetoxin binding being retained in solubilized sodium channel, gives us an excellent potential for isolating and identifying the brevetoxin binding component in excitable tissues.

IV. Conclusions

The brevetoxins bind to a unique receptor site associated with excitable membranes. The site, which possesses a K_d of 2.6-3.3 nM and a B_{max} of 5.75-6.0 pmoles/mg protein, is present in rat, fish, and turtle brain synaptosomes. This high affinity, low capacity site, is accompanied by a lower affinity, higher capacity site which is currently under investigation. The high affinity site is known as Site 5, and binds toxin with half-maximal affinity and avidity at concentrations which yield half-maximal ^{22}Na ion influx and half-maximal nerve membrane depolarization. Toxin binding at this high affinity site is membrane-potential independent and does not interact with any of the previously described sodium channel binding sites.

The binding characteristics of the lower affinity site correlate well with observed allosteric modulation observed in Sites 1-4 of the voltage-sensitive sodium channel. Photoaffinity probes incorporating brevetoxin in a covalent form displace brevetoxin from the binding site of high affinity, underscoring their potential as specific probes for purification of the site. Both size chromatography and brevetoxin-affinity chromatography, following solubilization of the site from membranes holds promise for isolation, purification, and characterization of the specific site. The Scope of Work outlined in the original contract has been completed.

V. Literature Cited in Summary

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VI. Chronological Bibliography of Publications Arising from Contract

A. Published

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B. In Press

Stuart, A.M., Baden, D.G. (in press) Binding of Florida red tide brevetoxins to voltage-dependent sodium channels of fish brain synaptosomes. J. Aquatic Toxicology.

Baden, D.G., Tomas, C.M. (in press) Variations in major toxin composition for six clones of *Ptychodiscus brevis*. Toxicon.

Baden, D.G. (in press) Public health aspects of red tides. 15 pp. In Handbook of Natural Toxins: Marine Toxins and Venoms V3 (A.T. Tu, ed) Academic Press, Inc. N.Y.

Baden, D.G., Mende, T.J., Szmant, A.M. (in press) 13 pp. In Lecture Notes on Coastal and Estuarine Studies. (M.J. Bowman, C.M. Yentsch, W.T. Peterson, eds.) Springer-Verlag, N.Y.

Trainer, V.L., Edwards, R.A., Szmant, A.M., Mende, T.J., Baden, D.G. (in press) 10 pp. Brevetoxins, unique activators of voltage-sensitive sodium channels. In Natural Toxins from Aquatic and Marine Environments. (S. Hall, ed) American Chemical Society Symposium Series.

Baden, D.G., Mende, T.J., Roszell, L.E. (in press) 4 pp. Detoxification mechanisms of Florida's red tide dinoflagellate *Ptychodiscus brevis*. In International Red Tide Symposium Proceedings. (T. Okaichi, T. Nemoto, D.M. Anderson, eds.) Elsevier Science Publishers.

Baden, D.G., Szmant, A.M., Mende, T.J., Trainer, V.L., Edwards, R.A. (in press) 4 pp. Brevetoxin binding to rat brain synaptosomes: Inhibition constants of derivatized toxins. In International Red Tide Symposium Proceedings (T. Okaichi, T. Nemoto, D.M. Anderson, eds.) Elsevier Science Publishers.

Baden, D.G., Tomas, C.M. (in press) 4 p. Variations in major toxin composition in six clones of *Ptychodiscus brevis*. In International Red Tide Symposium Proceedings. (T. Okaichi, T. Nemoto, D.M. Anderson, eds.) Elsevier Science Publishers.

Baden, D.G., Mende, T.J., and Trainer, V.L. Derivatized brevetoxins and their use as quantitative tools in detection. 8pp. 7th IUPAC Symposium on Mycotoxins and Phycotoxins, Elsevier Science Publishers, in press.

Baden, D.G., Mene, T.J., and Trainer, V.L. Derivatized brevetoxins and their use as quantitative tools in detection pp. Joint IUPAC/ICPP Proceedings on Mycotoxins and Phycotoxins. Soc. Mycotoxicol., in press.

C. Future Publication

Baden, D.G., Trainer, V.L., Edwards, R.A., Mende T.J. The binding of red tide brevetoxin PbTx-3 to rat brain synaptosomes: influence of membrane potential, derivative brevetoxins, and photoaffinity probe-linked brevetoxin on specific binding. Molecular Pharmacology.

Mende, T.J., Baden, D.G. Photoaffinity labels for defining receptor-ligand interactions. Tetrahedron Letters.

VI. Personnel Receiving Contract Support

A. Professional Personnel

Daniel G. Baden, Ph.D. P.I. [entire tenure of contract] 50% effort
Thomas J. Mende, Ph.D. Co-P.I. [entire tenure of contract] 20% effort
Andrew S. Gordon, Ph.D. Post-doctoral [7/85 to 9/85] 100% effort
Alina Szmant(Froelich), Ph.D. Asst Prof. [10/85 to 11/86] 50% effort

B. Staff

Laurie E. Roszell, B.A. Research Technician [5/86 to 1/87] 100% effort
Lloyd S. Schulman, Research Technician [1/87 to end] 100% effort

C. Students

Sally A. McNab, Undergraduate student [6/87 to 9/87] 100% effort
Andrea Morrison, High School student [9/85 to 12/85] 100% effort

D. Persons Receiving Graduate Degrees Related to Contract Research

Richard A. Edwards, M.S. Degree, Fall, 1988. [reference number 6 under Literature Cited].

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